Preliminary Study Protocol

The 3R Senolytic Approach — Version 2.0

Fisetin (Dose-Finding) + NMN + Spermidine in Pulsed Administration
Targeting Cellular Senescence in Healthy Volunteers
With Tissue Biomarkers (Skin Biopsy)

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August 2026 — Version 2.0

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1. Background — What is Cellular Senescence?

1.1 The "Zombie Cells" in Your Body

Imagine a cell that has been damaged by sunlight, pollution, or simply by living for a long time. In a healthy scenario, this damaged cell would repair itself or quietly die (apoptosis). But sometimes, the cell is too damaged to repair yet **refuses to die**. These are **senescent cells** — "zombie cells."

> 💡 **Simple Explanation**
>
> Senescent cells are like workers too tired to work but who won't retire. They sit at their desks doing nothing useful, and worse — they complain constantly, making everyone around them miserable.

1.2 How Does a Cell Become Senescent?

Every time a cell divides, protective caps called **telomeres** shorten. After ~50 divisions (the Hayflick Limit), they're too short and the cell stops dividing. Faster route: **DNA damage** from UV rays or oxidative stress activates **p53** → **p21** (temporary stop) → if damage persists → **p16^{INK4A}** (permanent stop) = senescence.

1.3 SASP — The Toxic Secretion

Senescent cells secrete the **SASP** (Senescence-Associated Secretory Phenotype): pro-inflammatory cytokines (IL-6, IL-8), proteases (MMPs that destroy collagen), and growth factors. The SASP is **contagious** — it pushes neighboring healthy cells into senescence. A snowball effect.

1.4 The Yoon Discovery (2018)

Yoon et al. took skin biopsies from brown spots and found: senescent fibroblasts with low **SDF1** (a protein that tells melanocytes to stop producing pigment) and high **tyrosinase** activity. When they eliminated the senescent fibroblasts, **the brown spots faded**. First experimental proof that senescent cells *cause* age-related pigmentation.

> **Key insight for our study:** Yoon measured biomarkers in *tissue biopsies*, not blood. If we want to validate their findings, we must do the same. Blood markers are supplementary, not primary.

2. Why This Study? — The 3R Hypothesis

The Three Rs

REMOVE — Kill the zombie cells

Fisetin (from strawberries) inhibits the SCAP anti-death network in senescent cells, triggering apoptosis. Healthy cells are unaffected.

REPAIR — Prevent new zombies

NMN boosts NAD+ (which drops 50% with age), restoring DNA repair enzymes (PARPs) and longevity proteins (sirtuins).

REGENERATE — Clean up

Spermidine induces autophagy — the cell's recycling system — to clear debris from killed zombie cells.

> 💡 **Simple Explanation**
>
> Demolish abandoned buildings (fisetin) → Fix damaged ones (NMN) → Clear the rubble (spermidine).

3. Study Design — 3-Arm Dose-Finding V2

3.1 Type of Study

Randomized, double-blind, placebo-controlled, 3-arm dose-finding trial.

> 💡 **Simple Explanation**
>
> **Randomized**: assignment by computer, not choice.
> **Double-blind**: neither participants nor researchers know who gets what.
> **3-arm dose-finding**: we test TWO different doses of fisetin to find the effective one. This is the key improvement in V2 — if only one dose fails, we learn nothing. With two doses, we can see a dose-response relationship.

3.2 Why 3 Arms Instead of 2?

V1 design (abandoned): Placebo vs. Fisetin 500mg. If 500mg doesn't work, we can't tell if the approach is wrong or just the dose is too low.

V2 design: Placebo vs. 500mg vs. 1,000mg. If 1,000mg works but 500mg doesn't, we've found the minimum effective dose. This is far more informative.

The 1,000mg dose is chosen because: (1) Mayo Clinic trials use up to 20mg/kg (~1,400mg for 70kg); (2) our piperine boost (×5-10 bioavailability) means 1,000mg + piperine already equals ~5,000-10,000mg standard fisetin — very ambitious but within safety margins.

3.3 Participants

Criteria	Details
Total number	45 volunteers (15 per arm)
Age range	50-75 years old
Arm A	15 people — Placebo
Arm B	15 people — Low dose (500mg fisetin)

Arm C	15 people — High dose (1,000mg fisetin)
All arms	NMN 250mg + Spermidine 3mg daily
Health status	Generally healthy

3.4 Duration

16 weeks (4 months), ~7 treatment pulses. Skin cell turnover = ~28 days, so 4 months = ~4 renewal cycles.

4. The Treatment Protocol

4.1 Three Groups

Component	Arm A (Placebo)	Arm B (Low)	Arm C (High)
Fisetin (pulse days)	0mg (cellulose)	500mg	1,000mg
Piperine (pulse days)	0mg	10mg	10mg
NMN (daily)	0mg (cellulose)	250mg	250mg
Spermidine (daily)	0mg (cellulose)	3mg	3mg

All capsules look identical. Arm A receives cellulose-filled capsules matching each schedule. Double-blinding is maintained by having an independent pharmacist prepare and label all capsules.

4.2 Pulse Calendar (16 weeks)

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Week 1-2: [FF] [.............] ← Pulse days 1-2 Week 3-4: [FF] [.............] ← Pulse days 3-4 Week 5-6: [FF] [.............] Week 7-8: [FF] [.............] Week 9-10: [FF] [.............] Week 11-12: [FF] [.............] Week 13-14: [FF] [.............] Week 15-16: [FF] [.............] ← Pulse days 13-14 (final) Total: 7 pulses × 2 days = 14 days of fisetin over 16 weeks NMN + Spermidine taken daily throughout.
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5. Biomarkers — Blood + Tissue V2

5.1 Why Tissue Biomarkers Are Essential

The biggest weakness of V1 was relying solely on blood p16/p21 from circulating leukocytes. These immune cells have different senescence dynamics than the fibroblasts, endothelial cells, and chondrocytes we actually want to target. Blood biomarkers tell us about systemic inflammation — but to validate the Yoon (2018) mechanism, we need to measure directly in skin tissue where the action happens.

5.2 Tissue Biomarkers (Skin Biopsy) — Primary Endpoints

Biomarker	Method	What It Tells Us
$p16^{INK4A}$	Immunohistochemistry (IHC) on 3mm punch biopsy	Direct count of senescent fibroblasts in tissue. Gold standard.
SA-β-galactosidase	X-Gal staining at pH 6.0 on frozen tissue	Blue staining = senescent cells. Visual, quantifiable.
SDF1/CXCL12	Immunofluorescence or tissue ELISA	The Yoon biomarker. Low SDF1 = senescent tissue. Should increase with treatment.
Melanin content	Masson-Fontana staining	Direct quantification of pigment. Should decrease with treatment.

Biopsy protocol: 3mm punch biopsy from sun-exposed forearm (dorsal surface). Local anesthetic (lidocaine). Two biopsies per participant: Day 0 and Day 120. Only for participants with visible solar lentigines (~60-70% of 50-75 age group). Suture or steri-strip closure, heals in 5-7 days.

5.3 Blood Biomarkers — Secondary Endpoints

Biomarker	Method	Expected Direction
p16/p21 mRNA	qPCR on leukocytes	↓ (but acknowledged as imperfect proxy)
IL-6, IL-8	ELISA	↓
NAD+	HPLC or NAD+ assay	↑

LC3-II/I, p62	Western blot	LC3 ↑, p62 ↓
SDF1 (serum)	ELISA	↑ (may not reflect tissue SDF1)
ALT, AST, GGT, bilirubin	Standard liver panel	Must stay normal (safety)

5.4 Measurement Schedule

Visit	Day	Blood	Biopsy	Clinical
1	0	✓ Full panel	✓ Baseline	✓ Full assessment
2	30	✓ Biomarkers + liver	—	Skin photos
3	60	✓ Biomarkers + liver	—	✓ Mobility + skin
4	90	✓ Biomarkers + liver	—	Skin photos
5	120	✓ Full panel	✓ Final	✓ Full assessment

6. Clinical Endpoints

Test	How	When
Brown spot area/intensity	Cross-polarized standardized photography	J0, J60, J120
Skin elasticity	Cutometer	J0, J120
6-Minute Walk	Standardized	J0, J60, J120
Grip strength	Hand dynamometer	J0, J60, J120
Gait speed (4m)	Timed walk	J0, J60, J120
Cognition	MoCA	J0, J120
Quality of life	SF-36	J0, J120
Adverse events	Daily diary + weekly calls	Continuous

Mandatory sunscreen: All participants (all arms) must use SPF 50+ daily to prevent new UV damage from confounding results.

7. Safety — Enhanced Monitoring V2

7.1 Inclusion / Exclusion

Include: 50-75 years, BMI 18.5-35, generally healthy, willing to provide blood AND skin biopsy.

Exclude: Active cancer, liver disease (ALT/AST >1.5× ULN), immunosuppressants, blood thinners, pregnancy, allergy to study compounds, current use of fisetin/NMN supplements.

7.2 Enhanced Liver Monitoring V2

> With 1,000mg fisetin + piperine, liver stress is the primary safety concern. Liver panel (ALT, AST, GGT, bilirubin) at **EVERY visit** (J0, J30, J60, J90, J120). Pre-specified stopping rule: if >2 participants develop ALT >3× ULN, study pauses for Safety Monitor review.

7.3 DSMB (Data Safety Monitoring Board) V2

An **independent Safety Monitor** (physician not involved in the study) reviews all adverse events at Day 30 and Day 60. They have authority to pause or stop the study. This is now mandatory because of the higher fisetin dose.

7.4 Ethics

- IRB approval required before enrollment
- Informed consent (explaining biopsy risks, supplement doses, right to withdraw)
- Registered on ClinicalTrials.gov before recruitment
- All data anonymized

8. Complete Equipment List

8.1 Lab Equipment

Item	Purpose	Est. Cost (USD)
Microplate reader	ELISA reading	$8,000-$15,000
qPCR machine	p16/p21 mRNA	$25,000-$45,000
Refrigerated centrifuge	Blood separation	$3,000-$6,000
-80°C freezer	Sample storage	$8,000-$12,000
-20°C freezer + 4°C fridge	Reagent storage	$1,500-$4,000
Micropipette set (P10-P5000)	Liquid handling	$600-$1,200
Vortex, heat block, pH meter	General lab	$600-$1,600
Precision balance (0.001g)	Encapsulation	$300-$800
Encapsulation machine (manual)	Filling capsules	$200-$600
Cryostat / access to pathology lab V2	Skin biopsy processing	$5,000-$15,000 (or outsourced)
Fluorescence microscope V2	IHC/IF on tissue	$5,000-$20,000 (or outsourced)

8.2 Consumables

Item	Est. Cost (USD)
ELISA kits (IL-6, IL-8, SDF1, NAD+, p62) × 5 timepoints	$3,000-$5,000
qPCR reagents + primers	$1,000-$2,000
Pipette tips, Eppendorf tubes, blood tubes, cryovials	$500-$1,000
Skin biopsy kits (3mm punch, lidocaine, sutures) V2	$500-$1,000
IHC/IH reagents (p16 antibody, X-Gal, Masson-Fontana) V2	$1,500-$3,000
Capsules (vegetarian, size 0) + cellulose	$100-$200

8.3 Supplements (45 participants × 16 weeks)

Compound	Quantity	Est. Cost (USD)
Fisetin (GMP, ≥98%)	~210g (15p × 14d × 500mg) + ~210g (15p × 14d × 1000mg)	$1,500-$3,000
NMN (GMP, ≥99%)	~840g (30p × 112d × 250mg)	$1,500-$3,500
Spermidine (GMP, ≥99%)	~10g	$400-$700
Piperine (95%)	~4.2g	$50-$100

8.4 Clinical + Other

Item	Cost
Camera + cross-polarized ring light	$800-$1,500
Dynamometer, BP monitor, scale, stadiometer	$400-$900
Software (R free, GraphPad optional)	$0-$500
Pathology outsourcing (if not in-house) V2	$10,000-$20,000
Safety Monitor fees V2	$2,000-$5,000
Personnel, IRB, participant compensation	$15,000-$30,000

8.5 Total Budget V2

Category	Est. Cost (USD)
Lab equipment (one-time)	$57,000-$122,000
Consumables + biopsies	$7,000-$13,000
Supplements	$3,500-$7,300
Clinical + pathology + DSMB	$28,000-$57,000
TOTAL V2	**$95,500-$200,000**
Follow-up studies (equipment already owned)	$25,000-$50,000

9. Timeline

Phase	Weeks	Activities
1. Preparation	-8 to 0	IRB, ordering, recruitment, screening, randomization, capsule prep
2. Treatment	1-16	7 pulses, 5 study visits (blood + 2 biopsies), weekly calls
3. Analysis	17-20	Lab analysis, unblinding, statistics, report

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Prep: [========] (Weeks -8 to 0) Treatment: [========================] (Weeks
1-16) Analysis: [====] (Weeks 17-20)
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10. Statistical Analysis

10.1 Primary Analysis

Compare tissue $p16^{INK4A}$ (IHC) between 3 arms at Day 120 using **one-way ANOVA** with post-hoc pairwise comparisons (Tukey HSD). This tests whether the number of senescent fibroblasts differs across groups.

10.2 Dose-Response V2

Linear trend test: Is there a dose-dependent decrease in p16 across placebo → 500mg → 1,000mg? This is the most powerful test for our 3-arm design. Even if neither dose reaches significance individually, a significant trend tells us the treatment is working and we just need more power.

10.3 Secondary Analyses

- Blood biomarkers: repeated-measures ANOVA across 5 timepoints
- Clinical endpoints: t-test or Mann-Whitney (Day 0 vs Day 120 change)
- Responder analysis: what % of participants show >20% reduction in tissue p16?
- Safety: frequency of adverse events by arm (Fisher's exact test)

10.4 Why 15 Per Arm Is Enough

With n=15 per arm and α=0.05, we can detect Cohen's d $\geq$ 0.9 with 80% power. This means large effects only. For a pilot study, this is appropriate — we're looking for signals, not definitive proof. If we see a dose-response trend (even if not individually significant), that justifies a larger Phase 2 study.

11. Glossary

Apoptosis: Programmed cell death. Senescent cells resist this.

Autophagy: Cellular recycling of damaged components.

Biopsy (3mm punch): Removing a tiny cylinder of skin (3mm wide) for analysis. Like a deep cookie-cutter. Heals in 5-7 days.

Cross-polarized photography: Photography through polarizing filters that eliminates surface reflections, revealing deeper skin structures.

DSMB: Data Safety Monitoring Board — independent experts who review safety data.

Dose-finding: A study design that tests multiple doses to find the effective one.

ELISA: Antibody-based protein detection using color change.

Fisetin: Natural senolytic from strawberries. Kills zombie cells.

HPLC: Molecule separation technique. Used for NAD+.

IHC (Immunohistochemistry): Using antibodies on tissue slices to detect specific proteins (like p16) directly in the tissue. Much more meaningful than blood tests.

IL-6 / IL-8: Inflammatory cytokines in the SASP.

Masson-Fontana: Silver staining that colors melanin black. Used to quantify pigment.

NAD+: Essential coenzyme that declines with age. Restored by NMN.

NMN: NAD+ precursor supplement.

$p16^{INK4A}$: Permanent cell-cycle stop protein. The gold-standard senescence marker.

SA-β-galactosidase: Enzyme overexpressed in senescent cells. Turns blue with X-Gal stain.

SASP: Toxic secretion from senescent cells.

SDF1: Protein that regulates melanocytes. Low in senescent tissue (Yoon 2018).

Spermidine: Natural autophagy inducer.

Tukey HSD: Statistical test for comparing multiple groups while controlling for false positives.

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